Exhibit 18

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Minireview

Production of pharmaceutical proteins from transgenic animals

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Abstract

Different systems are being studied and used to prepare recombinant proteins for pharmaceutical use. The blood, and still more the milk, from transgenic animals appear a very attractive source of pharmaceuticals. The cells from these animals are expected to produce well-matured proteins in potentially huge amounts. Several problems remain before this process becomes used in a large scale. Gene transfer remains a difficult and costly task for farm animals. The vectors carrying the genes coding for the proteins of interest are of unpredictable efficiency. Improvement of these vectors includes the choice of efficient promoters, introns and transcription terminators, the addition of matrix attached regions (MAR) and specialized chromatin sequences (SCS) to enhance the expression of the transgenes and to insulate them from the chromatin environment. Mice are routinely used to evaluate the gene constructs to be transferred into larger animals. Mice can also be utilized to prepare amounts as high as a few hundred mg of recombinant proteins from their milk. Rabbit appears adequate for amounts not higher than 1 kg per year. For larger quantities, goat, sheep, pig and cow are required.

No recombinant proteins extracted from the blood or milk of transgenic animals are yet on the market. The relatively slow but real progress to improving the efficiency of this process inclines to be reasonably optimistic. Predictive reports suggest that 10% of the recombinant proteins, corresponding to a 100 million dollars annual market, will be prepared from the milk of transgenic animals by the end of the century.

Key words: Pharmaceutical protein; Transgenic animal; Milk

1. Introduction

The preparation of recombinant proteins was one of the first successes of biotechnology, and more and more of these molecules are obtained and used each year. Indeed, recombinant proteins, including antibodies, are being used as pharmaceuticals for human and animal therapy (hormones, growth factors, antibodies), as antigens for vaccinations, as reagents in biochemical diagnosis, as enzymes in chemical industry, and as an experimental material in many research laboratories. Preparing recombinant proteins rather than native proteins from living organisms offers several advantages. In some cases, for example, erythropoietin, the protein is so rare in nature that the recombinant molecule is the only possible source for human therapy. In other cases, for example, insulin or human growth hormone, the recombinant is preferred to its counterpart from pig or human origin for safety reasons. More generally, genetic engineering most of the time offers the best way to obtain a given protein (and its mutants) in large amounts.

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Table 1
Some of the major post-transcriptionnal modifications of proteins

- Maturation of precursors (proteolysis)
- Cleavage of signal peptide
- Withdrawal of the methionine from NH_2 end
- Formation of S–S bridges
- Folding
- Glycosylation (O^- and N^-)
- Amidation
- Myristoylation
- Acetylation
- Phosphorylation
- Phenylation
- Association of subunits

The genetic code being universal, any gene from any origin can be theoretically expressed in the corresponding protein in any cell type as soon as the appropriate regulatory region has been associated with the coding part of the gene. The reality is in practise more complex. The vectors containing the gene to be expressed are in a certain number of cases of a poor efficiency, in an unpredictable manner. The recombinant proteins are sometimes not easily extractable from the reprogrammed cells. Moreover, a protein is not only a linear chain of amino acids. To be biologically active and stable in vivo, many proteins have to be properly folded and modified post-transcriptionally. A list of these modifications is summarized in Table 1. The cells reprogrammed with the foreign genes show variable capacity to synthesize, to mature and to secrete the corresponding recombinant proteins which they synthesize. For these reasons, several biological systems are being used and studied with the hope to improve them and, thus, to prepare large amounts of recombinant proteins at lower cost.

2. Why use transgenic animals?

The different systems which are being used to prepare recombinant proteins are listed in Table 2. Bacteria are easily reprogrammed, usually they synthesize the foreign proteins very efficiently, but are unable to perform some of the post-translational modifications, and particularly glycosylation, which takes place in the Golgi apparatus of eukaryotic cells. Most of the bacteria used until now do not secrete the recombinant proteins which are sometimes very difficult to extract (Blum et al., 1992). Theoretically, recombinant bacteria can synthesize as much pharmaceutical protein as desired.

Yeast is also easily reprogrammed and it can secrete proteins which are glycosylated. The enzymatic machinery which glycosylates proteins is, however, different in lower and higher eukaryotes, and yeast often secretes recombinant molecules inadequately glycosylated (Buckholz and Gleeson, 1991). As bacteria, yeast can theoretically be cultured in batches as numerous as wanted.

Fungi offer interesting possibilities for relatively large-scale preparations of recombinant proteins. The proteins prepared in this manner are not expected to have received appropriate post-translational modifications in all cases.

Transgenic plants can be obtained easily, at least for some species, and cultured in very large areas. They may be an ideal 'living fermentor' to prepare very large quantities of non-glycosylated proteins, such as human albumin (Sijmons et al., 1990).

In culture, animal cells can be easily reprogrammed using conventional transfection and infection with viral vectors. A certain number of cell lines can be maintained in fermentors and be the source of recombinant proteins. The post-translational modifications of the recombinant proteins prepared in this manner are usually quite similar to those observed in the native proteins. A

Table 2
The different systems producing recombinant proteins

	Amount	Extraction	Post-translational modifications
Bacteria	+ + + +	+ +	+
Yeast	+ + + +	+ + +	+ +
Fungi	+ + + +	+ + +	+ +
Transgenic plants	+ + + +	+ + ?	+ +
Baculovirus	+ + + +	+ + +	+ + +
Mammalian cells	+	+ + + +	+ + + +
Transgenic animals	+ + + +	+ + + +	+ + + +

Each of these systems has advantages and drawbacks roughly estimated by the cross in the table

relatively large variety of cell lines are already available for this purpose and their number can be increased after an immortalization of primary cells with oncogenes. Animal cells in culture thus offer virtually all the possibilities to synthesize bona fide recombinant proteins. The only limitation comes from the fact that animal cell culture remains relatively costly and cannot be easily developed on a very large scale as compared to bacteria or yeast. Hence, only recombinant proteins to be used in relatively small quantities, such as human erythropoietin, factor VIII or tissue plasminogen activator, are prepared from cultured animal cells (Datar et al., 1993).

Transgenic animals share most of the properties of animal cells in culture. They can properly carry out the post-translational modifications of recombinant protein. Cells in the animal are in an ideal metabolic situation and they can synthesize and secrete proteins very efficiently. These cells are as numerous as wanted as soon as transgenic animals are multiplied. Transgenic animals are, therefore, theoretically ideal fermentors. However, many problems remain to be solved before they can be used on a large scale.

3. Which transgenic animals to use

The first idea which comes to mind is to extract the recombinant proteins from the biological fluids of the transgenic animals (Table 3). In this respect, blood appears to be a good candidate. It is abundant and a low cost by-product of slaughterhouses. Many of the proteins secreted by the cells are found in the blood of animals. Hence, vectors driving the expression of the foreign genes in organs, such as liver or in most cell types of the animal, can be used. Such vectors are available. However, most of the blood proteins, at least those to be used for therapeutical purposes, are unstable and rapidly cleared from the circulation. These proteins have little chance to accumulate in blood in sufficient quantities to be used as the source of pharmaceuticals. This is the case for human growth hormone which has a half-life of about 15 min and which is, thus, not found at concentrations higher than a few $\mu g\ ml^{-1}$ in the blood of most transgenic mice, although their transgene is expressed at a high rate in many tissues under the control of strong promoters such as that of metallothionein gene (Palmiter et al., 1983). On the other hand, the recombinant proteins which are presently being prepared are essentially those having pharmaceutical properties in humans. Animals used for transgenesis, essentially mammals, are evolutionarily close to humans. The human recombinant proteins present in blood at high concentrations are expected to be active in the transgenic animals in most cases, and to alter more and less heavily the

Table 3
The different transgenic animals and biological fluids which can be used for the preparation of recombinant proteins

Source	Amount	Purification	Potential toxicity	Trans-genesis	Post-translational modifications	Specific advantages and drawbacks	Expected cost
Blood	+ + + +	+ +	+	+ +	+ + +	slaughterhouse by-product	+ +
Milk	+ + + +	+ + +	+ +	+ +	+ +	females only	+ + +
Egg white	+ + + +	+ + +	+ +	+	+ +	females only, transgenics not available	+ + +
Silk gland	+	+ + +	+ +	+	+	easy collection	+
Insect haemolymph	+	+ +	+ +	+ +	+	easy collection	+ +
Organ extracts	+	+	+	+ +	+ + + +	slaughterhouse by-product	+

health of these animals. This is indeed the case for growth hormone which induces giantism, diabetes and many other health troubles in transgenic mice, pigs and sheeps (Pursel et al., 1989). Moreover, blood is a relatively complex biological fluid. Preparing a recombinant protein from plasma may be not easy. It may also be particularly difficult to separate the human recombinant protein from the corresponding endogenous molecule. Blood can, however, be an interesting source of recombinant proteins in some particular cases. Massoud et al. (1991) were able to obtain transgenic rabbits expressing the human α_1-antitrypsin gene in their liver. The human protein was found in the blood of the animals at a concentration as high as 1 mg ml^{-1}. Such an accumulation was possible not only because the transgene worked actively, but certainly also because this protein is naturally highly stable in blood and present at an elevated concentration in blood of animals. It is also for this reason that the transgenic rabbits were perfectly healthy. The human α_1-antitrypsin extracted from the blood of the rabbits was glycosylated and biologically active. Another striking example is the case of human haemoglobin which can be synthesized in an active form in the red blood cells of transgenic pigs (Swanson et al., 1992). Human factor VIII was also found, although at a relatively low level, in the blood of transgenic mice (Mikkelsen et al., 1992). Interestingly, a DNA fragment containing the core region of human immunoglobulin κ locus gave rise to the secretion of functional antibodies in transgenic mice obtained from ES cells previously transfected with a YAC vector containing the foreign DNA (Davies et al., 1993).

Egg white is thought to be an excellent source of recombinant proteins. It is abundant, easily collected and the strong promoter of ovalbumin gene should be able to very efficiently drive the expression of a foreign gene in the oviduct of transgenic chicken. Although attractive, this system has gained little attention so far. This is obviously due to the fact that it remains a very difficult task to prepare transgenic birds for technical reasons (Shuman, 1991). Yolk, although also abundant, cannot so easily be the source of recombinant proteins. Indeed, vitellogenin is synthesized in liver, transported by blood, absorbed by the oviduct where it contributes to form yolk.

The secretions of silk gland and insect haemolymph are possible candidates as sources of recombinant proteins (Maeda et al., 1985; Rancourt et al., 1990). The availability of these biological fluids is, however, limited, and it is by no means certain that the recombinant proteins elaborated by the cells of these non-vertebrates will be properly matured.

It may happen that a given protein is properly matured in only a very limited number of cell types of an animal. In such a situation, the recombinant molecule has little chance of being present in blood or in another abundant and easily collectable biological fluid in sufficient amounts to be purified at an industrial scale. In such cases, it is conceivable to collect organs generating the recombinant proteins in their appropriate form from the transgenic animals in slaughterhouses and to start to purify the proteins from these tissues.

At present, milk appears to be the best source of recombinant proteins. It is very abundant, very rich in proteins, easily collected, and not expected to contain toxic substances. Mammary gland secretion is directed essentially towards the outside of the body, being devoted to offsprings. A recombinant protein secreted in milk is, therefore, not expected to interfere greatly with the metabolism of the lactating female. A few years ago, Simons et al. (1987) gave a direct demonstration that this method is most likely amenable to the industrial area when they observed that transgenic mice secreted up to 23 mg ml^{-1} of ovine β-lactoglobulin in their milk. For these reasons, most of the efforts to use transgenic animals as the source of recombinant proteins lie presently with milk.

4. Which vectors to use

4.1. The available protein gene regulatory regions

As mentioned above, the vectors to be used for directing the expression of transgenes in a given tissue or in all tissues must contain the appropri-

ate regulatory regions. Many of such DNA sequences are now available, showing acceptably good specificity for a given cell type and, in some cases, a high potency. In cases of recombinant proteins being prepared in the milk of transgenic animals, the regulatory regions from milk protein genes must be used (Fig. 1). These genes are expressed at high levels exclusively in the mammary gland of lactating animals. Their regulatory regions are thus expected to direct the expression of a foreign gene specifically in this tissue. The major specific milk proteins are α_{s1}-, α_{s2}-, β- and κ-caseins present in most species at various concentrations, β-lactoglobulin present essentially in ruminant milk, α-lactalbumin synthesized in most species, and whey acidic protein (WAP) found essentially in rodent milk. These genes are studied for several reasons: to know their structure to improve animal genetic selection, to use them to tentatively change milk composition (to improve cheese yield or to humanize milk), to use their promoters to produce recombinant protein in milk and, of course, to do fundamental studies on the hormonal control of their expression. The milk protein genes from different species inducing human, cow, goat, sheep, pig, camel, rabbit, guinea pig, rat, mouse and even marsupials are being studied (Campbell et al., 1984; Qasba and Safaya, 1984; Jones et al., 1985; Hall et al., 1987; Gorodetski et al., 1988; Ali and Clark, 1988; Vilotte et al., 1989; Thépot et al. 1990, 1991; Devinoy et al., 1991; Koczan et al., 1991; Jolivet et al., 1992a,b; Persuy et al., 1992; Lee and Oka, 1992; Groenen et al., 1992; Schmidhauser et al., 1992; Roberts et al., 1992). A relatively large number of DNA sequences to be used for the generation of vectors expressing foreign genes, specifically in the mammary gland, are thus available.

4.2. The level of transgene expression in milk

Experiments carried out by the various groups are listed in Tables 4–6. From these data it appears that when fragments of genomic DNA containing the whole milk protein genes were used to generate transgenic animals, the expression of the transgenes was very high in several cases, reaching the level observed with endogenous genes (Table 4). With gene constructs containing the whole foreign gene and a milk protein gene regulation sequence, the level of expression was high in some cases and very low in others. It was particularly high in the case of human α_1-antitrypsin gene in mouse and sheep using ovine β-lactoglobulin gene promoter (Archibald et al.,

Table 4
Expression of whole milk protein genes from various origins in transgenic animals

Gene	Transgenic animal	Protein (mg ml^{-1})	Ref.
Ovine-β lactoglobulin	mouse	23	Simons et al., 1987
Ovine-β lactoglobulin	mouse	0–23	Harris et al., 1990
Rat-β casein	mouse	a few	Lee et al., 1988
Goat-β casein	mouse	24	Persuy et al., 1992
Goat-β casein	mouse	1	Roberts et al., 1992
Bovine-α lactalbumin	mouse	0.45	Vilotte et al., 1992
Bovine-α lactalbumin	mouse	3.7	Soulier et al., 1992
Rat WAP	mouse	a few	Bayna and Rosen, 1990
Rat WAP	mouse	a few	Dale et al., 1992
Mouse WAP	pig	1	Wall et al., 1991
Mouse WAP	pig	1	Shamay et al., 1991
Mouse WAP	pig	1	Shamay et al., 1992a
Mouse WAP	pig	1	Shamay et al., 1992b
Mouse WAP	mouse	not measured	Burdon et al., 1991a
Mouse WAP	mouse	not measured	Burdon et al., 1991b

The values indicated as mg ml^{-1} of milk represent the highest concentration found in all the animals of a given experiment.

1990; Wright et al., 1991) of human and bovine growth hormone and the Arg 358 mutant α_1-antitrypsin genes (Bischoff et al., 1992; Devinoy et al., 1993). It was very low with the human PS2 gene using the mouse WAP promoter (Tomasetto et al., 1989) and with the human erythropoietin using the rabbit WAP promoter (Attal et al., unpublished data). When foreign cDNA were used rather than whole genes, the expression was low in almost all cases. Curiously, the human protein C was expressed at a relatively high level ($\cong$ 1 mg ml^{-1}) in the milk of transgenic pigs and very poorly in transgenic mice, although the mouse WAP promoter was in the gene construct (Velander et al., 1992).

From the data, it is difficult to draw clear general rules to construct efficient vectors for expression of foreign genes in milk. Most likely, the regulation regions from the various genes are not of equal potency. This is probably the case for the WAP promoters. Indeed, the mouse WAP gene promoter was rather weak when associated with the whole human growth hormone gene (Reddy et al., 1991; Tojo et al., 1993), whereas the rabbit WAP gene promoter was particularly potent with the same gene (Devinoy et al., 1993). The rabbit promoter region used contained a 6.3-kb DNA fragment, whereas the mouse promoter region only used a 2.6-kb DNA fragment. An essential stimulatory element was found at about 6 kb in the rabbit promoter (Devinoy et al., 1991). This element is most likely not present in the mouse promoter, which might explain its relative weakness. As a general rule, the regulatory elements involved in the control of milk protein gene expression are still far from being known in detail. The gene constructs using these promoters are therefore still done empirically, leading to unpredictable success and failure.

4.3. The role of introns in transgene expression

The regulatory elements located upstream of the transcribed regions of the genes are of course not the only controlling factors in the overall expression of a gene. The different elements which may interfere with the gene transcription, the mRNA stabilization and translation are depicted in Fig. 2. Introns which are often of no use in expressing foreign genes in cultured cells, even when integrated, are essential in most cases in transgenic animals (Brinster et al., 1988). Obviously, introns do not all have the same intrinsic potency. The intron SIS formed with one part of an immunoglobulin gene intron and another part of an adenovirus gene intron showed very high potency (Choi et al., 1991), whereas the SV40 small t intron is not fully functional due to its extremely small size (Fu and Manley, 1987; Huang and Gorman, 1990). However, the intrinsic potency of an intron is not the sole factor responsible for its activity when added in a gene construct. Indeed, a systematic study carried out by Palmiter et al. (1991) revealed that a given intron

Table 5
Expression of foreign whole genes in the milk transgenic animals

Regulation region	Foreign gene	Transgenic animal	Secreted protein (ml^{-1})	Ref.
Ovine-β lactoglobulin	human-α_1 antitrypsin	mouse	7 mg	Archibald et al., 1990
Ovine-β lactoglobulin	human-α_1 antitrypsin	sheep	35 mg	Wright et al., 1991
Mouse WAP	human PS2	mouse	1.5 μg	Tomasetto et al., 1989
Mouse WAP	human GH	mouse	500 μg	Reddy et al., 1991
Mouse WAP	human GH	mouse	a few μg	Günzburg et al., 1991
Rabbit WAP	human GH	mouse	22 mg	Devinoy et al., 1993 Stinnakre et al., 1992
Rabbit WAP	bovine GH	mouse	8 mg	Devinoy et al., in press
Rabbit WAP	human-α_1 antitrypsin-Arg 358	mouse	10 mg	Bischoff et al., 1992

The concentration of the foreign proteins in milk shown in the table are the highest found in the animals of a given experiment.

may or may not be efficient according to the cDNA and the promoter to which it is associated. It seems, therefore, that introns contain multiple signals of unknown nature which govern the status of the gene during development. The association of a given intron with the rest of the gene construct must also create cryptic signals, case by case, which modify more or less heavily the expression of the resulting transgene. Therefore, in most cases the presence of at least one intron seems to be compulsory to obtain a good expression of a transgene. However, in this respect no general rules can be edicted as long as more combinations will have been obtained and examined. Unfortunately, cultured cells provide only limited information about the efficiency of a gene construct to be used in transgenic (Brinster et al., 1988).

The mechanisms leading to elimination of intron sequences from pre-mRNAs are not fully understood, but important facts have been reported. Proteo-ribonuclear complexes are involved in splicing. These complexes recognize sequences along the mRNA which are located in both sides of the internal exons. An internal exon must, therefore, not exceed 300 nucleotides in length to allow association of the splicing complex with the pre-mRNA (Robberson et al., 1990).

The first and last introns seem to be properly withdrawn only if the proteo-ribonuclear complexes recognize the CAP structure and the AAUAAA polyadenylation signal, respectively (Ohno et al., 1987; Niwa and Berget, 1991). A specific consensus sequence may also greatly contribute to eliminating a given intron when present in the following exon (Watakabe et al., 1993). The insertion of a foreign cDNA into a gene which proved efficient in transgenesis turned out to give very disappointing results in most cases (McClenaghan et al., 1991). This strategy relies on the idea that a DNA sequence which showed high efficiency as a transgene must contain indispensable elements for expression. The insertion of a foreign cDNA is not expected to alter this

Table 6
Expression of foreign cDNAs in the milk of transgenic animals

Regulatory region	Foreign cDNA	Transgenic animals	Protein (ml^{-1})	Ref.
Goat-β casein	human CFTR	mouse	a few μg	Di Tullio et al., 1992
Bovine-α casein	ovine trophoblastin	mouse	1 μg	Stinnakre et al., 1991
Bovine-αs_1 casein	human lactoferrins	cow	?	Krimpenfort et al., 1991
Ovine-β lactoglobulin	human serum albumin	mouse	2.5 mg	Shani et al., 1992
MMTV	bovine-αs_1 casein	mouse	0.3 mg	Yom et al., 1993
Ovine-β lactoglobulin	human factor VIII	sheep	?	Halter et al., 1993
Ovine-β lactoglobulin	human γ-interferon	mouse	20 ng	Dobrovolsky et al., 1993
Bovine-αs_1 casein	human t-PA	mouse and rabbit	50 μg	Riego et al., 1993
Mouse WAP	human β-interferon	mouse	?	Schellander and Péli, 1992
Mouse WAP	human t-PA	mouse	460 ng	Gordon et al., 1987
Mouse WAP	human t-PA	mouse	460 ng	Pittius et al., 1988
Mouse WAP	human t-PA	goat	3 μg	Ebert et al., 1991
Mouse WAP	human CD4	mouse	200 ng	Yu et al., 1989
Mouse WAP	human protein C	pig	1 mg	Velander et al., 1992
Ovine-β lactoglobulin	human factor IX	sheep	25 ng	Simons et al., 1988
Ovine-β lactoglobulin	human factor IX	sheep	25 ng	Clark et al., 1989
Ovine-β lactoglobulin	human α_1- antitrypsin	mouse and sheep	18 mg	McClenaghan et al., 1991
Bovine-αs_1 casein	human urokinase	mouse	2 mg	Meade et al., 1990
Rabbit-β casein	interleukin-2	rabbit	430 ng	Bülher et al., 1990
Rat-β casein	human FSH	mouse	15 μg	Greenberg et al., 1991

The concentration of the foreign proteins in milk are the highest found in the animals of a given experiment.

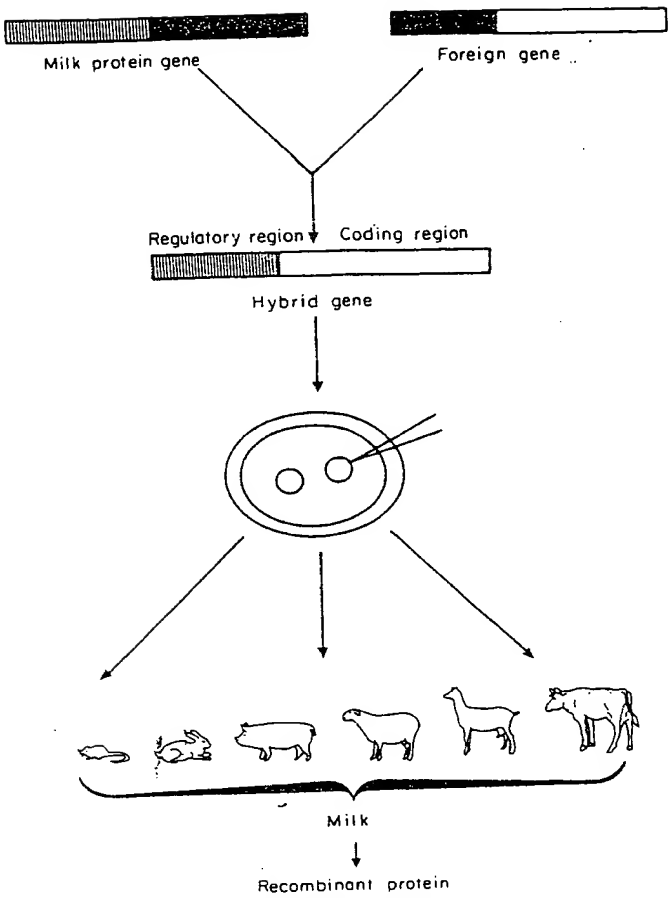

Fig. 1. Schematic representation of the generation of vectors for the expression of foreign genes in the milk of transgenic animals. The regulatory region from a milk protein gene can be fused to the transcribed region of a gene of interest. The resulting gene construct can be injected into embryo pronuclei to generate transgenic mammals of various sizes which are expected to express the foreign gene in their milk.

efficiency. In practice, the reverse is in most cases the reality. Generally speaking, ideally a whole gene with its own elements selected during evolution should therefore be used rather than the corresponding cDNA. Unfortunately, in some cases only cDNA are available or in practise amenable to easy manipulation. This is true for factor VIII gene which is as long as 250 kb. The low level of expression obtained when cDNAs are introduced into whole genes used as vectors is most likely due to the fact that the above-reported rules have not been respected.

4.4. The influence of untranslated regions of mRNA

Essentially the same reasoning is true for the other elements of the gene constructs. The 5′ untranslated region (5′ UTR, also named leader) may favour more or less translation. A minimum length of 77 nucleotides is required to obtain maximum efficiency (Kozak, 1991). A leader sequence rich in GC is generally less efficient than another richer in AU (Kozak, 1991), the GC structures being more stable, preventing the ribosomes to reach the initiator AUG with good efficiency (Gallie et al., 1987). The 5′ UTR may even contain major elements drastically controlling the translation of the mRNAs. This is the case for several iron-binding proteins which have a regulatory element activated by the metal ion (Klausner and Harford, 1989).

The 3′ UTR of mRNA participate in some cases in the stabilization of the mRNA. The mRNA for some house-keeping genes and particularly for some oncogenes have AU-rich sequences in their 3′ UTR which make them fragile towards nucleases. Quite different mechanisms are in fact involved in the control of mRNA stability (Cleveland and Yen, 1989). These facts have to be taken into consideration in the construction of a vector for transgenesis.

4.5. The influence of transcription terminator

Transcription termination is carried out through mechanisms which have not been completely elucidated (Edwalds-Gilbert et al., 1993). Empirically, it appears that different terminators have quite different intrinsic potency. The termi-

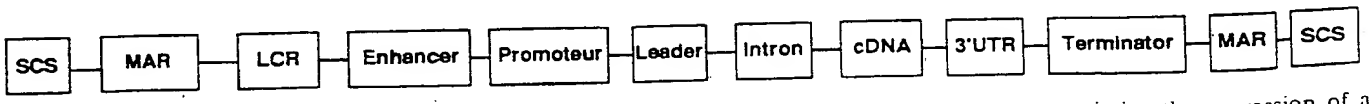

Fig. 2. Schematic representation of the various elements which should be present in a vector to optimize the expression of a transgene. For details see the text.

nator of SV40 late gene is thus 4-times more potent that its early gene counterpart (Carswell and Alwine, 1989). A short DNA sequence (≅ 70 bp) in the mouse WAP gene terminator appeared more efficient than a longer one of several kb in transgenic mice, whereas the late gene SV40 terminator, which is highly potent in cultured cells, worked very poorly in these conditions (Dale et al., 1992). The human erythropoietin gene contains in its terminator region a silencer which is more or less activated according to oxygen tension (Semenza and Wang, 1992). Choosing an efficient transcription terminator cannot at present be done following logical rules. Moreover, it would not be surprising if a given terminator exhibits quite different efficiency according to the construct in which it is inserted.

4.6. The influence of transgene insertion site

Experiments carried out with transgenic animals soon revealed that in the great majority of cases, a transgenic expression is heavily dependent on its site of integration in the host genome rather than on the number of copies. The site of integration of a transgene is presently unpredictable when the DNA is microinjected into the pronuclei by the conventional method. This largely explains why the expression of a transgene in a given animal is also unpredictable. This observation was done repeatedly with transgenes expressed in the milk with one clear and noticeable exception. The whole β-lactoglobulin gene was not only expressed at a high rate in transgenic mice, but also as a function of its copy number (Whitelaw et al., 1992). This DNA fragment might thus contain a MAR or a SCS (see below). The fact that the expression of a given transgene is governed by its chromatin environment has been ascertained by experiments in which a given transgene, weakly and strongly expressed in different mice, was isolated from these animals and reinjected into new mouse embryos. The resulting transgenic animals expressed their transgene at a high and low level when they received the efficient and inefficient mouse genomic DNA containing the transgene, respectively (Al Shawi et al., 1990).

The idea which is generally accepted is, therefore, that the transgene must be located in a highly transcribed open region of the chromatin to work properly. Apart from being obtained by chance, such a situation may be encountered, at least to some extent, using appropriate vectors. Genomic DNA fragments which were able to allow a good expression of a given transgene (Al Shawi et al., 1990) might be used to construct vectors. This rather heavy approach does not seem to have been retained so far. Some genes isolated or belonging to a given group of genes are controlled by locus control regions (LCR). This is the case for the human β-globin locus. Such a DNA sequence is located far upstream of the globin genes. This sequence, which is DNAse I hypersensitive, must be added to the isolated globin genes from this locus to allow their expression as transgenes (Stamatoyannopoulos, 1991).

The β-globin LCR acts as a super enhancer which strongly stimulates the transgenes which are then expressed according to their copy number. Until now only a very limited number of LCR have been identified. Each LCR seems to be specific in a given locus and it is by no means certain that each gene or group of genes is dependent on such regulatory elements. The whole bovine casein locus can be isolated in DNA fragments not longer than 200 kb (Ferretti et al., 1990; Threadgill and Womack, 1990). The presence of an LCR specific in this casein locus has not been reported so far. Such a sequence inserted into vectors might, of course, improve specifically the expression of foreign genes in milk.

Chromatin is bound to the nuclear structure called matrix, forming loops. DNA is linked to the nuclear matrix through sequences named matrix attached regions (MAR) or scaffold attached regions (SAR). Many, but not all, of these sequences are AT rich (Dworetzky et al., 1992; Jankelevich et al., 1992). Some of them are involved in the control of DNA replication and segregation (DePamphilis, 1993). Others (or possibly the same MAR) play an essential role in controlling gene expression (Bonifer et al., 1991; Zhao et al., 1993). Some MARs have been proven to have the capacity to insulate a gene and a

transgene from their chromatin environment leading to a high expression which becomes dependent of their copy number (Bonifer et al., 1990). MAR may thus be added on one end, and perhaps preferably on both ends, of a gene construct (one MAR may perhaps be sufficient when concatemers of the injected material are formed before its integration. In these conditions, the transgene becomes surrounded by MAR). A coinjection of conventional gene constructs and MAR may lead to the generation of transgenics harbouring the foreign gene in the vicinity of the cointegrated MAR. This cointegration of the foreign DNA and the MAR results in a significant improvement in the expression of the transgene (McKnight et al., 1992). This simple procedure does not oblige the experimentators to introduce in vitro MAR sequences in their gene constructs. The exact role of MAR is in fact presently somewhat confusing. The presence of an AT-rich region with the ATTA and ATTTA consensus sequences appear to favour greatly the expression of downstream genes (Bode et al., 1992; Boulikas, 1993; Schöffl et al., 1993). However, an AT-rich consensus sequence which is capable of binding strongly to nuclear matrix may be unable of stimulating the expression of a transgene. Such a situation was observed when the MAR sequence located in the downstream region of the human apolipo-protein B100 (Levy-Wilson and Fortier, 1989) was added in front of the rabbit WAP or the cytomegalovirus early genes promoters (Attal et al., unpublished observations).

Recent data suggest that sequences capable of insulating genes and transgenes are located in the upstream region of the human and chicken β-globin locus (Chung et al., 1993) and of the mouse metallothionein gene (Palmiter et al., 1993). These sequences seem to not necessarily have nuclear matrix-binding capacity, and they show strong functional analogy with AT-rich regions named SCS (specialized chromatin structure) which have been found in drosophila genome and which strongly insulate transgenes (Kellum and Schedl, 1991; Farkas and Udvardy, 1992).

SCS regions rather than MAR sequences should therefore be added at the beginning and perhaps at the end of a gene construct to generate efficient insularity of transgene. Confusion may have come from the fact that the A element located in the upstream region of chicken lysozyme gene has the capacity to insulate a transgene (Bonifer et al., 1990), while also being a MAR (von Kries et al., 1990).

An open configuration of chromatin in the vicinity of a given transgene can be obtained by coinjection of the gene construct of unknown efficiency and a gene known to become regularly an efficient transgene. Using this simple method, a construct containing the β-lactoglobin promoter fused to the human α_1-antitrypsin cDNA became better expressed in transgenic mice when injected with the entire ovine β-lactoglobin gene (Clark et al., 1992). It is not known whether a sequence expressed specifically at a high level in mammary cells is required to obtain this rescuing of transgene expression or whether any highly expressed sequence might have the same effect. One may hypothesize that a gene not specifically expressed in milk will stimulate the expression of the gene construct but also will be responsible for a significant expression in tissues other than the mammary gland.

Vectors, the YAC, being expressed in yeast, can harbour very long fragments of DNA, up to several hundreds of base pairs. The YAC can thus be used to generate vectors containing long fragments of genomic DNA surrounding milk protein genes. These long fragments have a good chance of containing most, if not all, the elements which control the expression of the genes and the transgenes. YAC constructs proved to be utilizable in generating transgenic mice which express their transgenes in a satisfactory manner (Capecchi, 1993; Davies et al., 1993).

4.7. Use of embryonic stem cells (ES) to generate transgenic animals

Embryonic stem cells can be cultured and reintroduced into developing embryo and participate to the generation of chimaeric animals. Foreign genes can be introduced with a homologous recombination into the genome of the ES cells before they are mixed with the cells of the devel-

oping embryo (Bradley et al., 1992). This complex procedure leads to the precise replacement of one gene by another. In this manner, a foreign DNA sequence can be introduced within a milk protein gene. As expected, the foreign gene introduced with this method will be well-expressed. However, this approach remains restricted to mice, ES cells from other species being not presently available. The possibility of using embryonic germ cells (EG cells) rather ES cells to generate chimaeric animals (McLaren, 1992) has recently opened new avenues for farm animals which are the only species to be potential producers of recombinant proteins at an industrial scale.

5. Which mammals to use

Mammals vary quite differently in size, and several of them have been chosen to produce recombinant proteins in their milk. Ruminants, namely goat and sheep, appear the best candidates to produce proteins up to several tons per year (Ebert et al., 1991; Wright et al., 1991). Pig is considered as a possible living fermentor, although milk cannot be collected as easily as from ruminants (Velander et al., 1992).

Rabbit produces up to 200–250 ml of milk per day. Its milk is particularly rich in protein and a significant proportion of milk can be obtained with a simple device (Lebas, 1970; Duby et al., 1993). Transgenic rabbits can be easily obtained at a relatively low cost. This species is also highly prolific and it is therefore a good candidate for the production of recombinant proteins not exceeding 1 kg per year.

Table 7
The potential production of recombinant proteins in the milk of different transgenic mammals

Animal	Amounts of proteins to produce in milk
Mouse	up to 1 g
Rabbit	1 kg a^{-1}
Sheep, goat, pig	1–1000 kg a^{-1}
Cow	several t a^{-1}

Mice do not easily deliver their milk. Incubating the isolated mammary gland on ice for a few hours leads to a complete secretion of the milk it contains. Undegraded and biologically active human growth hormone was obtained in this way (about 1.5 ml per mouse; Stinnakre et al., 1992). Following this procedure, 100 lactating mice might provide a few hundred mg of a recombinant protein, a quantity which is sufficient to study the biochemical and some of clinical properties of the protein. Transgenic mice can therefore be considered not only as laboratory animals to evaluate the efficiency of gene constructs to be used in larger species but as a true 'living mini-fermentor'.

Cow is probably the only mammalian species potentially capable of synthesizing the 400 t of human albumin which are needed each year (Table 7). Generating transgenic cows using the conventional gene microinjection is a very burdensome and costly task. This is due to the fact that much less embryo can be obtained in a given period of time by the conventional superovulation and in vivo fertilization classically used in mouse.

Table 8
Comparison of transgenic yield in different species

Species	Number of embryos per superovulation	Rate of pregnancy (%)	Number of donors per recipient	Rate of born embryos (%)	Transgenic animals (% of born)	Number of females per transgenic	Months to obtain F2
Mouse	15	50	2	10–20	15	10	7.5
Rabbit	20	50	2	10	10	15	17
Pig	15	40	2	5–8	10	20	38
Sheep	4	40	1–5	15	5	40	52
Cow	5	20	1	10	5	40	100

The yield of transgenic calves is also generally very low in comparison to other species (Table 8).

It has become possible to obtain one cell embryos through an all in vitro protocol. This method includes the following steps: collection of ovaries from females at any physiological stage in slaughterhouse, in vitro maturation of oocytes isolated from the ovaries, and in vitro fertilization of the oocytes. The availability of embryos is considerably increased with this procedure. Consequently their cost is dramatically reduced. This method has been defined and even used in cow (Krimpenfort et al., 1991). It has been extended to sheep and is being adapted to goat. After the gene microinjection, the cow embryos can be cultured up to the blastocyst stage. Only the embryos surviving the manipulation reach this stage. A detection of the transgene in a few cells explanted from the blastocytes can be performed using the PCR technique, although with somewhat limited reliability (King and Wall, 1988; Ninomiya et al., 1989; Burdon and Wall, 1992). Using all these techniques, no more donor females are required to obtain embryos and a minimum number of recipients are needed to develop the embryos considered as transgenic after the PCR test. Thanks to these techniques, transgenesis in farm animals has been greatly facilitated and the use of milk as the source of recombinant has consequently become closer to reality.

6. What is the future of the 'living fermentors'?

Predicting what will be the real future of living fermentors is not easy. A few years ago several reports indicated that by the end of this century 10% of the recombinant proteins would be extracted from the milk of transgenic animals, representing an annual market of approx. 100 million dollars (Genetics Technology News, 1990; Technology Management Group, 1990). The reality is that there is still no protein prepared from transgenic animals on the market. Human α_1-antitrypsin is well on the way and protein C might follow. It seems, therefore, hard to believe that the situation described above will be encountered so quickly. Indeed several problems remain not completely solved. Transgenesis in farm animals is being greatly improved by the new techniques of embryo manipulation (Fig. 3). Obtaining transgenic farm animals will, however, remain a rather hard task with relatively long delays between the time when a gene construct is available and the

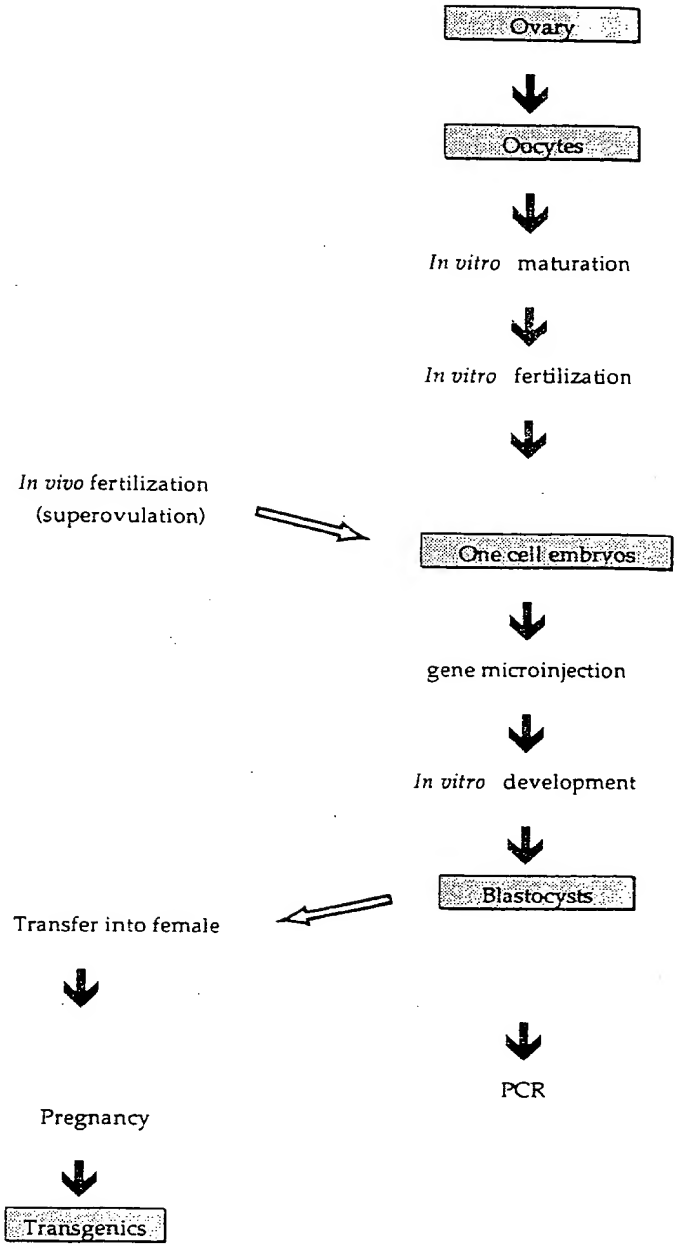

Fig. 3. Schematic representation of the different steps of the protocol leading to the preparation of mature oocytes, one cell embryos and blanstocysts in vitro. For details see the text.

moment a significant number of animals can be milked. The possibility to clone the embryo through nuclear transfer (Yang and Anderson, 1992) will help to shorten the delay, allowing a by-passing of a part of the reproduction cycle.

The construction of efficient vectors turned out to be more complicated than anticipated. This is due to the fact that the problem is in itself complex. During embryo development a transgene is subjected to many events which may lead to an inactivation of the chromatin region in which it is integrated. Testing new constructs to be expressed in milk is a slow process. Indeed, cell cultures are of little help for this purpose (Brinster et al., 1988) and transgenic animals must be obtained to test each vector. Only transgenic females can be directly used and the information concerning the vectors becomes available only when they are mature and lactate. In fact, a relatively small number of laboratories seem to be concerned with vectors expressing foreign genes at a high level in transgenic animals. Essentially, people trying to produce recombinant proteins or to express antisense RNA presently need potent vectors. For the great majority of studies using transgenic mice, vectors working modestly are sufficient to express such or such cellular protein of interest. Although the construction of efficient vectors for expression of foreign genes in milk is not a problem solved in all cases, there are some reasons to believe that, in future, perhaps 80% of the transgenes will work in a satisfactory manner. Until this situation becomes a reality, if it does, success is welcome but cannot be predicted.

Purifying foreign proteins from milk does not seem a major problem. Milk is not an extremely complex biological fluid. It does not degrade proteins through proteolysis. A satisfactory biochemical purity of the recombinant proteins seems to be obtained with conventional methods (Ebert et al., 1991; Wilkins and Velander, 1992). In most cases, the recombinant protein is expected to be present in a soluble form in the whey. Interestingly, in one case at least, a hydrophobic protein, the CFTR, was found associated with the membranes surrounding the milk lipid globules (Di Tullio et al., 1992). The hydrophobic proteins can thus be recovered from milk fat. Most likely, however, their content will be much lower than that of water-soluble proteins, the surface of the lipid globule membranes harbouring the recombinant proteins being relatively limited.

The biological purity of the recombinant proteins extracted from milk will probably be more difficult to ascertain. Virus and prions may still contaminate the purified recombinant proteins. Tests will have to be done to detect such contaminants and the herds of transgenic animals will have to be kept under excellent breeding conditions. It is indeed paradoxical to use genetic engineering to prepare recombinant proteins in order to escape the use of human organ but still to come back to transgenic animals to obtain these molecules. Some long-standing practises incline, however, to be optimistic. Indeed, insulin injected daily to diabetics has long been exclusively extracted from pig pancreas without being highly purified. Still, this hormonal preparation is considered safe, as far as viral contamination is concerned.

It is by no means certain that the mammary epithelial cells are able to proceed to all the desired post-translational modifications. These cells can indeed be O- and N-glycosylated proteins and they are differentiated for efficient secretion of proteins in contrast to most commonly used recombinant cells. However, milk proteins which are glycosylated are known to contain somewhat variable amounts of carbohydrates, as though the glycosylation process could not be quantitative, given the high flow of neo-synthesized proteins which migrate through the Golgi apparatus. Glycosylation of proteins is known to depend on the physiological state of the cells and the microheterogeneity of glycoproteins may modify, to some extent, their biological properties (Goochee and Monica, 1990).

In this respect, however, the recombinant proteins secreted in milk are not expected to be less exploitable than those found in the medium of cells expressing foreign genes. Moreover, it is conceivable to genetically modify the mammary cells through transgenesis with genes coding for glycosylation enzymes to give them the capacity to glycosylate proteins in a more appropriate

manner. Although theoretically feasible, as it is for animal cells in culture and yeast, such cellular modifications may, in practise, more or less profoundly disturb the cell physiology.

The post-translational modifications of proteins are not the only problem which may limitate the use of transgenic animals to produce recombinant proteins. The process of migration in the cell and exocytosis of the neosynthesized problems is complex. It involves the action of Golgi apparatus and it seems that signals within the protein structures more or less favour the migration of proteins towards the outside of the cells (Edington, 1992). In this respect, it is interesting to note that the human factor VIII in its ΔII deleted form is more readily secreted than the natural molecule, as though some sequences of these proteins contributed to slow its exportation out of the cells (Meulien et al., 1988).

Although milk is secreted in a specific compartment not directly in contact with blood circulation, one may expect that some of the recombinant proteins will cross the mammary epithelium. Indeed, caseins are not present in blood during lactation but a protein like WAP is obviously crossing the mammary epithelium and it is found at a significant level in the circulation (Grabowski et al., 1991). It is conceivable that only the smallest proteins will pass through the epithelium, but in these cases, the transgenic animals may be profoundly disturbed by the presence of an active recombinant protein in their blood when they start lactating. One may thus predict that, in a certain number of cases, the animals will not be utilizable. Lactation itself may be profoundly altered by the product of the transgene as it is for WAP expressed too early, during pregnancy in mouse and pig (Burdon et al., 1991a,b; Shamay et al., 1992a,b).

On the other hand, most of the transgenes show a background of aspecific expression in tissues other than those in which they should be expressed. This is also the case for foreign genes fused to milk protein gene promoters. It is generally admitted that this is due to the fact that the transgenes are integrated in the host genome in the vicinity of endogenous regulatory regions which reduce more or less their specificity of expression. This problem may be solved by choosing the transgenic lines of animals which show the lowest leaky expression out of the mammary gland. The addition of SCS in the vectors may also contribute to reduce the aspecific expression of the transgene (McKnight et al., 1992). Whatever happens, the flow-through of some recombinant proteins from milk to blood will, in some cases, remain an inevitable event. A point of some potential importance must also be taken into consideration. Blood or a given organ can be considered as slaughterhouse by-products but only of course if meat remains the major product. Given the difficulties encountered by companies which propose transgenic plants for human consumption, one way predicts that selling the meat from transgenic animals at a normal price will be a real problem, at least the first time. The rejection of genetically modified organisms by some people is undoubtedly in most cases more emotional than reasonable. Reasonably, it is imaginable that acceptance of 'transgenic' meat will increase in the future after becoming more familiar with the situation (Van Brunt, 1990; Fox, 1993a,b). During this transition period, a company may be forced to delay its use of transgenic animals to produce recombinant proteins. The resulting cost may dissuade the use of 'living fermentors' for some time. A similar, although much less acute problem, may be encountered when the recombinant proteins are being produced in milk. Indeed, the consumption of the transgenics by human beings may also be forbidden in this case but the resulting extra costs are expected to be minor, given the relatively reduced number of involved animals.

Improvement of the techniques aiming at producing recombinant proteins from transgenic animals are real and this approach remains very attractive, given its very high potency, once the transgenic animals have been generated. Of course, the real commercial success of this method will be greatly determined by its cost. Indeed, the other biological systems capable of producing recombinant proteins and, in particular, transgenic plants (Hiatt, 1990; Mason et al., 1992; Hamamoto et al., 1993) are also being improved (Hodgson, 1993). An insufficient number of recombinant

proteins of commercial interest has been prepared from animals to allow a precise comparison with the competition systems and particularly with cultured mammalian cells. The course of progress inclines therefore to remain reasonably optimistic.

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